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Ghost peaks observed after AP-MALDI experiment may disclose new ionization mechanism of matrix assisted hypersonic velocity impact ionization

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Abstract

RATIONALE—Understanding the mechanisms of MALDI promises improvements in the sensitivity and specificity of many established applications in the field of mass spectrometry. This paper reports a serendipitous observation of a significant ion yield in a post-ionization experiment conducted after the sample has been removed from a standard atmospheric pressure (AP)-MALDI source. This post-ionization is interpreted in terms of collisions of microparticles moving with a hypersonic velocity into a solid surface. Calculations show that the thermal energy released during such collisions is close to that absorbed by the top matrix layer in traditional MALDI. The microparticles, containing both the matrix and analytes, could be detached from a film produced inside the inlet capillary during the sample ablation and accelerated by the flow rushing through the capillary. These observations contribute some new perspective to ion formation in both laser and laserless matrix-assisted ionization.

METHODS—An AP-MALDI ion source hyphenated with a three-stage high-pressure ion funnel system was utilized for peptide mass analysis. After the laser was turned off and MALDI sample was removed, ions were detected during a gradual reduction of the background pressure in the first funnel. The constant-rate pressure reduction led to the reproducible appearance of different singly- and doubly-charged peptide peaks in mass spectra taken a few seconds after the end of the MALDI analysis of a dried-droplet spot.

RESULTS—The ion yield as well as the mass range of ions observed with a significant delay after a completion of the primary MALDI analysis depended primarily on the background pressure inside the first funnel. The production of ions in this post-ionization step was exclusively observed during the pressure drop. A lower matrix background and significant increase in relative yield of double-protonated ions are reported.

CONCLUSIONS—The observations were partially consistent with a model of the supersonic jet from the inlet capillary accelerating detached particles to kinetic energies suitable for matrix-assisted hypersonic-velocity impact ionization.

Introduction

Together with electrospray ionization (ESI)^{[1],[2],[3]} and atmospheric pressure chemical ionization (APCI), matrix-assisted laser desorption ionization (MALDI)^[4] is a mainstream method used in analytical mass spectrometry (MS) for converting neutral molecules into

positive ions through protonation reactions. These three ionization methods are usually used separately but sometimes in combination in a wide variety of analytical applications for the mass analysis of proteins, peptides, and small molecules. In the last decade, MALDI mass spectrometry (MS) has become indispensable in the important new application of tissue imaging.^[5] While the mechanisms of ESI and APCI are understood in some detail, the underlying mechanism of MALDI is still hotly debated.^{[6],[7]} Recently, discussion of the elementary processes in MALDI has shifted from gas-phase proton exchange between neutral analytes and the protonated matrix molecules,^[8] the latter being protonated through two-photon absorption or exciton energy pooling mechanism. An alternative and more reasonable model considers the escape of protonated analyte ions from hot molecular clusters^{[9],[10],[11]} or nanoparticles which are produced in abundance during laser ablation.^[12] In this cluster paradigm,^{[13],[14],[15],[16]} the MALDI process starts with laser-assisted ablation and is completed with the release of protonated analytes from matrix clusters; the last stage is believed to be governed by processes similar to those responsible for ion desolvation in ESI.^[17] A major problem in MALDI models is to explain the charge separation that accompanies the ablation. The appearance of positive charge on MALDI clusters could be explained, for instance, by the ejection of electrons from irradiated MALDI crystals or desorbed nanoparticles,^[18] the electrons being generated through photo-induced processes.^[19] The charging of ablated particles can also be attributed, particularly in case of IR MALDI from solid matrices, to thermionic emission^[20] or fracto-emission^{[21],[22]} as observed in catastrophic fracturing of solids. Even three decades after the invention of MALDI, separation of charges in MALDI is still a disputed topic.

A key characteristic of MALDI is the threshold laser energy required to see any ion yield; noting that this laser energy is absorbed by a thin top layer of the sample. In addition, at laser energies above the threshold, the ion yield depends almost exponentially on the absorbed energy. This would suggest that MALDI, in essence, is a thermal process, with an Arrhenius-like dependence of the ion yield on the sample temperature. The dependence of the MALDI threshold on the sample temperature,^[23] combined with recent studies on the in-source decay of peptides in MALDI,^[24] indicates that thermal activation should be considered a major characteristic of the MALDI mechanism.^{[25],[26]}

To estimate the energy-per-molecule ratio required for MALDI ions to be produced, one can take $E_{th} = 10^3 \text{ J/cm}^3$ as an energy density threshold for the appearance of peptide ions in MALDI.^[6] In general, the threshold value will depend on the nature of matrix and on the wavelength of the UV laser. It is useful to note here that the same estimate of $E_{th} = 10^3 \text{ J/cm}^3$ is obtained in the case of the popular CHCA (α -cyano-4-hydroxycinnamic acid) matrix when recalling that, first, the laser fluence producing sufficient peptide ion signals can be as low as 10 mJ/cm^2 for this matrix^{[27],[28],[29]} and secondly, the depth that laser radiation penetrates into the matrix crystal is as small as $0.1 \mu\text{m}$. This penetration depth can be obtained from considering that 337-nm laser radiation is almost entirely absorbed at $1/k$ depth where k is the absorption coefficient, approximately 10^5 cm^{-1} for CHCA^[30]. This rather substantial energy ($E_{th} = 146 \text{ kJ/mol}$, given the CHCA molecular weight (MW) of 189 Da and its density, 1.3 g/cm^3), is larger than the CHCA sublimation enthalpy (100 kJ/mol ^[31] or 1 eV/molecule) and it is deposited in a very short time and into a very thin

layer on a top of a MALDI crystal. This introduces another important feature of the MALDI process: the formation of a large number of nanoparticles.

The important factors affecting the ablation threshold are the absorbed energy and the rate of thermal energy diffusion from the irradiated sample volume during the short laser pulse. The energy absorbed by the top layer is less than the laser energy directed onto the MALDI crystal due to partial reflection caused by a mismatch in refractive indices, and also because the laser illuminates the facets of crystals at different, often oblique, angles. Furthermore, during laser irradiation, the thermal energy absorbed by the top layer ($<0.1\ \mu\text{m}$ in thickness) is diminished by heat dissipation into the underlying sample material. The influence of sample surface morphology on the yield of MALDI ions is known. It directly affects the above threshold measurements because even during a short (5–10 ns) laser pulse the absorbed energy spreads into the depth of the crystals thus affecting the surface temperature rise, the instance of phase explosion, and the eventual ablation of the top layer.

Accordingly, obstruction of heat dissipation from the top layer leads to a comparatively higher rate of temperature rise and, in its turn, to a higher desorption/ionization rate. This effect has been observed during the irradiation of micron-sized MALDI crystals at low laser energy levels^[32]. Results of calculations on heat transfer presented in the same study showed that at the same laser energy fluence, submicron crystals evaporate exponentially faster than crystals of much larger size. Further evidence that reducing the size of microcrystals significantly increases MALDI efficiency can be derived from experiments where solutions contained both MALDI matrices and nanoparticles or graphite microparticles;^{[33],[34],[35]} such a mixture after drying resulted in the formation of smaller and more homogeneous crystals on MALDI plates. These studies indicate that when the size of individual particles becomes similar to the heat conduction length, the MALDI threshold decreases. Combining these observations reminds us of the obvious: it is the energy that is absorbed and retained in the top layer of individual microcrystal during the short laser pulse that determines a true MALDI threshold. It is worthwhile to mention at this point that the effect of obstructed heat transfer in pulsed laser ablation from metal or semiconductor layers of variable thickness has been studied for decades.^[36] MALDI threshold experiments conducted in line with the above ablation studies would be very illuminating to the study of the influence of sample thickness, and of sample morphology in general, on the yield of analyte ions in MALDI. However, these experiments would require a controlled growth of homogeneous matrix crystals of known thickness, which is not very easy to accomplish.

Additional evidence that the MALDI threshold for submicron crystals can be significantly lower is provided by studies on LDI from nanostructured surfaces.^[37] It was shown that the ion signals of 3-methoxybenzylpyridinium desorbed from nanowires were detected at a laser energy density 4-fold lower than required for the same ions to be produced during laser irradiation of standard dried-droplet MALDI samples. In conclusion, one can expect that under conditions where the heat does not significantly dissipate from the irradiated micron-size crystals during a short laser pulse, the MALDI threshold can be over a factor of 2 lower than with samples where the crystal size is in the 10–50 μm range.

Protonation, in contrast to photoionization or electron ionization, provides much softer ionization for most biological molecules with basic groups. The process of proton exchange between a proton donor (e.g., MALDI matrix) and a proton acceptor (a basic group) has a much lower energy barrier than photo- or surface ionization. In the absence of laser heating of the sample, the physico-chemical conditions that lead to protonation can be achieved if one “re-enacts” MALDI, yet without the laser. This hypothetical MALDI-like process can involve a rapid energy deposition into preformed submicron particles causing not only thermal but also an immense mechanical stress leading to disintegration of those particles. Achieving a rapid heating and shattering of microparticles on a time scale of 3–5 nanoseconds (i.e., time that is equal to a typical duration of laser pulses in MALDI), can result in the above- mentioned fracto-emission, creating charged analytes and nanoparticles.

There exists an often-overlooked method of ionizing small molecules known as hyperthermal surface ionization.^[38] In this method, molecules as large as fullerenes^[39] are first evaporated or laser-desorbed from solid materials^[40] into an atmosphere of nitrogen or helium at 0.5–5 bar pressure. Then this gas seeded with the molecules is expanded into a vacuum chamber through a nozzle. A few mm away from the nozzle, these molecules gain a supersonic velocity. Finally in the vacuum chamber, the accelerated molecules hit a solid surface with a large work function, such as tungsten oxide, and are ionized. Hyperthermal surface ionization has been demonstrated for sodium atoms accelerated to supersonic velocities.^[41] The efficiency of hyperthermal surface ionization for both atoms and molecules increases exponentially with increasing kinetic energy of the impinging particles. In the acceleration of heavy molecules by supersonic jets, the extent of the entrainment, and therefore the acceleration efficiency, depends on the number of collisions with the gas molecules that the molecule experiences when passing through the nozzle and on the mass difference between the gas (nitrogen, helium or hydrogen) and the seed (the analyte).^[42] It has been shown experimentally that the terminal velocity for organic molecules with medium masses can easily reach 1000 m/s^[43] in nitrogen and even higher velocity in helium jets.

The mechanism of hyperthermal surface ionization in its simplest form can be explained as the conversion of the kinetic energy of the impinging molecule into its electronic energy. When the molecule hits the surface, its kinetic energy $E = mv^2/2$ (or in eV, $E_k = 0.5 mc^2(v/c)^2$ where c is the speed of light) is transferred into electronic energy and added to the work function of the surface, thus reducing the effective ionization energy. From a quantum mechanical viewpoint, a valence electron of the molecule striking the surface jumps to the surface from its ground state over the reduced energy barrier (the reverse process can be impeded due to asymmetry in the crossing energy levels in Landau-Zener mechanism).^[44] The molecular mass, expressed in eV/c², can be estimated as the number of nucleons contained in a given molecule (i.e., nominal mass M) multiplied by the average nucleon mass $m_N = 0.94 \times 10^{-9}$ eV/c². For example, an anthracene molecule with a mass $M = 178$ Da accelerated to a hypersonic velocity of 3,000 m/s (10^{-5} of the speed of light) has a kinetic energy of 8.4 eV. Acceleration to such a high energy undoubtedly results in high-efficiency hyperthermal ionization of anthracene molecules, and hyperthermal surface ionization has been demonstrated for anthracene, fullerenes, and a few other small, yet, “robust”

molecules. Unfortunately, this method, similarly to electron ionization or resonant laser ionization, does not provide “soft ionization” of larger molecules such as peptides but causes their dissociation.

In the late 1980s, acceleration to high velocities in supersonic jets was shown to be effective not only for molecules but also for micron-size particles. For instance, in a process called gas dynamic cold spray^[45] particles with diameters between 5 and 50 microns were accelerated to 1 km/sec velocity^[46] even at atmospheric conditions. In this cold spray technique, the aerosol or particulate matter is sprayed using a supersonic nozzle from a chamber filled with a gas kept at 10 atm or higher pressure towards the target kept at atmospheric conditions.^[47] Essentially the same phenomenon, yet referred to as vacuum kinetic spraying (VKS),^[48] was used to accelerate ultrafine particles (< 1 micron) by a supersonic jet expanding into a low-vacuum.^{[49],[50]} In VKS, due to lower decelerating drag downstream of the supersonic nozzle, the submicron and nanoparticles can travel with 1 km/s and higher velocities to a target located 10–100 cm from the nozzle.

As can be inferred from the above discussion, MALDI-like conditions can be attained in a process similar to VKS when the clusters or nanoparticles of MALDI matrix with embedded analytes hit a solid surface. The previous studies on cold spray and VKS showed that in energetic collisions the micron-size particulate matter can shatter, almost instantly, producing hot and much smaller particles as well as clusters. Consider a $1\mu\text{m} \times 1\mu\text{m} \times 1\mu\text{m}$ CHCA particle aerodynamically accelerated to 1.2 km/s (approximately Mach 3.5). The kinetic energy of this particle is $0.6 \cdot 10^{-9}$ J and the energy density is $0.6 \cdot 10^3$ J/cm³. This value is higher than the threshold observed in LDI of small molecules desorbed from nanowires and close to the MALDI threshold for submicron crystallites. When brittle microparticles collide with a solid surface, the concentrated focus of kinetic energy and consequent explosive release of thermal energy along with mechanical shattering can cause the appearance of charged clusters with temperatures above 500 K, which is the principal feature of the MALDI process.^[51] Finally, it is worth noting that high-velocity impact ionization is routinely observed when space microdust moving with an orbital velocity of 10 km/sec collides with the surface of a satellite.^[52] Recently, a hypervelocity impact ionization has been demonstrated for submicron polymethylmethacrylate particles accelerated to a 4–8 km/s velocity by means of high-voltage electrostatic system.^[53] Over a decade ago, molecule-related ions (oligonucleotides and peptides) were observed in the high-velocity impact of accelerated aqueous microparticles with a target surface.^[54] A jet airplane moving with subsonic speed through a cloud with ice crystals will acquire high static charge on its surface due to the same phenomenon.

In this paper, a combination of an ion funnel and a linear trap mass spectrometer was used to detect signals from singly- and doubly-charged peptide ions a few seconds after the completion of MALDI analyses of dried-droplet samples. The reproducible appearance of ion signals in the absence of laser irradiation could be explained by a mechanism of matrix-assisted hypersonic-velocity impact ionization.

EXPERIMENTAL

Chemicals and sample preparation

The three peptides used in this study: Bradykinin fragment 1–7, monoisotopic molecular weight (MMW)=756.400 Da, Angiotensin II, MMW=1045.542, and Pro14-arg, MMW=1532.858 Da were purchased from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions of the peptides were prepared at 10^{-3} M concentration in water with 0.1% trifluoroacetic acid (TFA). A purified α -cyano-4-hydroxycinnamic acid (CHCA) used as the MALDI matrix was purchased from Bruker (Billerica MA, USA). Acetonitrile, water, methanol, and TFA were purchased from Sigma-Aldrich.

An ESI tuning mix G2431A/G2431 from Agilent (Santa Clara CA, USA) producing singly-charged ions of hexakis phosphazene with m/z values of 322, 622, 922, 1522, was used for mass calibration and LTQ/funnel performance optimization in the ESI experiments. A CHCA matrix solution was prepared at 0.3 mg/mL in 70% acetonitrile/29.9% water/0.1% TFA (v/v/v) and then used to make a stock solution with a peptide concentration of 0.1 pmole/ μ L. Two dozen microdroplets, each containing 1 μ L of this mixture, were deposited onto the surface of a 0.8-mm thick stainless steel MALDI plate (Optiplat-196, ABI, Framingham, MA, USA). Immediately after deposition of the last droplet, the MALDI plate was placed into a freezer kept at -20° C. After 30 minutes, the plate was taken out of the freezer and placed into a box at room temperature. The spots dried over 15 minutes. A microscope inspection of the dried droplets revealed 10–15 μ m microcrystals homogeneously spread over a circular spot with a diameter of 1.5-mm.^[55] The spots containing dried droplets were cold washed with water.^[56]

Mass Spectrometer with the ion funnel

A linear ion trap mass spectrometer (LTQ, Thermo Fisher, San Jose CA, USA) was used in all experiments. It was equipped with a standard AP-MALDI source (MassTech Inc., Columbia MD, USA). The source utilized a 355-nm UV laser pulsing at 200-Hz. The conical inlet of the LTQ used to heat the 1.6-mm o.d. stainless steel capillary was removed from the LTQ entry flange and a triple-stage ion funnel with orthogonal injection (Spectrograph, Richland, WA, USA)^[57] was mounted on the same flange. The ion funnel inlet held a 0.75-mm i.d., 1.6 mm o.d., 85-mm long stainless steel capillary. The minimum pressure in the first funnel achieved with this large-bore capillary was 3.5 Torr. In ion transmission studies at lower pressures, this capillary was substituted with a 0.38 mm i.d. stainless steel capillary. The o.d. and length of the narrow-bore and large-bore capillaries were the same. The temperature of the capillary head (approximately one third of the capillary length) varied from 100° C to 165° C. Such a low temperature, compared with the much higher temperatures (300 – 350° C) typically used in AP-MALDI systems was due to a use of a Viton o-ring that provided vacuum isolation of the capillary. A 60-mm long section of the inlet capillary extended into the low-pressure chamber with the funnel. This section of the stainless steel capillary was heated only by thermal conductance along the capillary body. Thus, the average temperature of the capillary was even lower than 165° C.

The AP-MALDI system was equipped with an optical fiber to deliver the UV radiation from the laser output lens to the focusing lens in the AP-MALDI source, which focused the laser radiation into a 300- μm spot. The LTQ was operated in automated gain control (AGS) mode. In the MALDI experiments, the maximum injection time was kept at 100 ms. The laser pulse energy was adjusted to a low level (approximately 15–20 μJ), which was slightly above the AP-MALDI threshold (~ 10 μJ) for obtaining peaks with high signal-to-noise (S/N) ratios in all the experiments.

The ion funnel comprised three stages. Figure 1S and the corresponding captions (Supporting Information) describe the design and operation of all three “flow through” funnel stages. In addition, the design of the third stage, the funnel trap, has been described in detail elsewhere.^{[58],[59]} Synchronization of the ion funnel trap operation^[60] with the injection cycle of the LTQ mass spectrometer showed approximately a several-fold increase in the efficiency of ion usage in AP-MALDI LTQ MS experiments because the ions constantly generated by the AP MALDI source enter the funnel trap and are stored there during the 160-ms time interval when the mass scan is performed in the LTQ, although the trapping mode was not used in this study.

In the funnel equipped with orthogonal sample injection, the inlet capillary was inserted through a cut in a sidewall of the first funnel cylindrical section (Fig. 1S, Supporting Information). Because the printed circuit board (PCB) sidewall of the funnel was nearly airtight, the rectangular cut provided the main route for gas evacuation from the interior space of the first funnel. The 1-mm thick front plate covering the axial entry to the first ion funnel had two slits providing an additional air evacuation route. A small area in the funnel sidewall opposite to the capillary nozzle was adapted to provide an additional gas evacuation route: the gaps between the adjacent PCBs in this area were opened by removing sections of the isolating plastic sheets.

The first stage was evacuated using a separate roughing pump equipped with a throttle valve that allowed the pressure inside the first-stage funnel to be controlled between 3 Torr and 16 Torr, while keeping the pressure p_{00} inside the LTQ ion transporting system (flatapole section RF₀₀) within the limits ($p_{00} < 0.1$ Torr) specified by the manufacturer.

The front plate was maintained at 380 V, and the exit plate of the first funnel at 200V. The capillary was held at 300 V. The voltage drop across the second funnel was 100 V. The DC voltage applied across the exit of the third funnel was -6 V. This DC voltage was varied from -6 V to -10 V to compare signals during ion transmission studies. An RF generator supplied 180-V voltage (peak-to-peak) at 650-kHz frequency to the PCB ring electrodes in the first funnel and another RF generator supplied 120-V voltage (peak-to-peak) at 850-kHz frequency to the PCB ring electrodes in the second and third funnels. The gas pressure in the first funnel was measured using a convectron gauge/controller system (300 Series, Kurt L. Lesker, Jefferson Hill PA, USA). The gas pressure in the second and third funnels was measured by the LTQ pressure monitoring system.

RESULTS AND DISCUSSION

Ion funnel transmission

The operation of the funnel was optimized with a nanospray ionization source (Flex, Thermo Fisher). The commercially-available solution of the Agilent calibration mix was sprayed at a rate of 0.3 $\mu\text{L}/\text{min}$ through a 10- μm i.d. fused silica tip (New Objective, Woburn, MA, USA) into the 0.75 mm i.d. inlet capillary of the triple stage funnel system. The recorded ESI spectrum and chemical noise are shown in Fig. 2S (Supporting Information). The distance between the ESI tip and inlet was varied from 0.5 cm to 10 cm. The linear trap mass spectrometer (LTQ) was operated in AGS mode, with the maximum injection time set as 10 ms. No change in the total signal intensity was observed when the tip-to-capillary distance varied, which was a striking contrast to the pattern observed during regular LTQ operation (i.e., without the ion funnel) where a reduction in the ESI signal intensity is typically observed at tip-to-capillary distances larger than 1 cm.^[61] This indicates that the air intake by the 0.75 mm i.d. capillary is significantly larger than that in the LTQ system equipped with the 0.5 mm i.d. inlet capillary, which improves the collection of microdroplets from the highly divergent ESI plume. A remarkable 0.3%/hour stability (see Fig. 3S in Supporting Information) was demonstrated for the intensity of the dominant peak (referred to as NL or normalization level in the Thermo Fisher Excalibur software). It is worth mentioning that using fresh high-quality fused-silica tips for the ESI emitters greatly improves the ion signal stability. The fresh 10- μm i.d. fused silica tip demonstrated a high stability for several hours.

In an AP-MALDI experiment, the target plate with samples was placed 2.15-mm from the opening of the 0.75 mm i.d. capillary serving as the inlet to the ion funnel. The DC voltage applied to the plate was 4.4 kV. No change in heights of the observed m/z peaks was detected when the pulsed delay focusing (PDF)^[62] in the AP-MALDI source was switched from “on” to “off” regimes, which indicates that a substantial fraction of the MALDI plume including the ions was drawn into the capillary by the strong flow of inrushing air. Measurements conducted under similar MALDI source conditions showed that the triple-stage ion funnel operating without use of the synchronized trapping mode improved the throughput of the MALDI mass analysis by a factor of 5–7, compared with a standard LTQ system configuration. The increase in signal, nevertheless, did not propagate into better signal-to-noise ratios due to the proportional increase in chemical background. Because the effect of chemical noise generated by MALDI sources can be dramatically reduced using ultra high-resolution instruments ($R > 100,000$), the use of ion funnels will benefit MALDI methods developed for such instruments. Another way to diminish chemical noise is to operate the inlet capillary at much higher temperatures. The AP-MALDI sources are used both with low-end ion trap instruments (e.g., the LTQ system from Thermo Fisher or the 6300 ion trap from Agilent) and with high-end mass spectrometers (e.g., the Orbitrap from Thermo Fisher). All the above instruments are equipped with nozzle-skimmer subatmospheric interfaces utilizing 80–100-mm long 0.5 mm i.d. capillaries heated to 350°C. Heating the capillary to 350°C provides effective desolvation of MALDI ions and reduces chemical noise compared with similar inlets kept at room temperature.^[63] In the present funnel design, the highest temperature of the capillary was limited to 165° C; in

addition, the capillary was only 85 mm long and had a much larger i.d. The latter two factors prevented the heat from capillary walls spreading inward to the center of the capillary,^[64] thus limiting heating of the particulate matter travelling through the center. Using rectangular capillaries (for example, 0.4 mm by 2 mm) can provide more effective heating of microparticles travelling through the capillary while keeping high gas intake.

Figure 1 shows the transmission of ions generated by the ESI source utilizing the 10- μ m i.d. fused-silica tip spraying the Agilent calibration mix through the triple-stage funnel at several different pressures in the first funnel. The pressure levels in the second and third stages read from the LTQ pressure monitoring system were a factor of 8 lower than those in the first funnel. The ion transmission was calculated by summing the intensities of peaks with m/z 322, 622, 922, 1522. In these experiments, the axial field in the tapered part of the third stage was varied by changing the voltage applied to its exit orifice. It was found that the maximum ion transmission was attained when the background pressure in the first funnel reached 13 Torr (1.6 Torr in the second/third funnels); the transmission of ions through the funnel system only slightly diminished between its maximum and 16 Torr. At higher pressures, the turbomolecular pump could not provide the necessary vacuum in the multipole ion guide section of the LTQ system. It can be seen also from Fig. 1 that by increasing the axial field in the tapered section of the third funnel, the ion transmission noticeably increased at pressures lower than 12.5 Torr.

In another experiment, a use of the 0.38 mm i.d. inlet capillary allowed the same pressure to be established in all three funnel stages by throttling the LTQ and funnel roughing pumps. Figure 2 shows the pressure dependence of the ion transmission for individual ion signals from the calibration mix through the triple-stage funnel system kept at constant background pressure. Comparing Figs. 1 and 2, one can draw a conclusion that a significant ion loss takes place in the second and third funnel stages (both these stages are differentially pumped by the LTQ roughing pump) when the pressure in these stages drops below 0.5 Torr. In the next experiment, the pressure in the first funnel was kept high ($p_1=14$ Torr) and the pressure in the second and third stages was kept at the optimum value ($p_{2,3}=1.75$ Torr), and the DC voltages in the first funnel were then varied. It was noticed that up to 30% change in the axial field inside the first funnel caused less than 10% change in the intensity of ion signals from the calibration mix. This indicates that the air flow is a significant factor that helps to move ions from the first to the downstream sections of the ion funnel system. In general, gas dynamics is known to be an important factor affecting the ion flux from the high-pressure funnel towards the lower pressure downstream section of the mass spectrometer. In a study conducted over 10 years ago, it was shown that high-pressure funnels can transmit the ions to downstream ion guides kept at lower pressures even without axial fields^[65].

Recent modeling of the funnel with parameters close to those utilized in this study demonstrated that the supersonic jet issuing from the capillary nozzle into the funnel kept at a few Torr pressure retains a very high speed even at a distance of several cm^[66]. The particles entrained into such a jet form a narrow beam that can travel a substantial distance inside the funnel under study.^[67] To understand the transport of nano- and microparticles in the funnel with the orthogonal injection, one needs to take into account the interaction of the gas jet with a background gas and eventually with the opposite wall. The ion transmission

can be modeled taking into account the aerodynamic drag as well as the RF, DC, and space charge forces exerted on the ions. This necessitates using accurate modeling of the gas jet parameters using computational fluid dynamics (CFD) software.^[68]

AP-MALDI experiments

Figure 3 demonstrates a typical mass spectrum obtained in the analysis of 0.1 pM samples at a laser energy level of 15 μJ , 50% above threshold (8–10 μJ). This energy threshold is typical for a standard AP-MALDI source where the UV laser is focused into a 300- μm spot onto the surface of a MALDI plate and the laser spot is aligned with the axis of the LTQ inlet capillary. Note that contrary to vacuum MALDI, AP-MALDI has a broader range of laser energies where the in-source decay does not overwhelm signals from intact molecule-related ions. Figure 3 shows the doubly-protonated pro14-arg peak (m/z 767.4); its height is approximately 10–15% that of the singly-charged peak (m/z 1533.8).

Figure 4S (Supporting Information) shows representative background noise patterns in six different m/z regions of the AP-MALDI spectrum. The spectrum was taken under conditions where the laser energy was approximately 3 times the threshold value; it is a sum of 5,000 individual LTQ acquisition scans (approximately 3 scan/s). Figure 5S (Supporting Information) shows the mass dependence of the average intensity of the chemical noise peaks, all singly-charged, determined for different m/z regions. It can be seen that this dependence is in good agreement with the $1/m$ trend^[69] reported earlier for the intensity of background noise observed in MALDI. These results, especially for low m/z regions ($m/z < 190$) where no contribution from matrix clusters or matrix adducts is expected, support the suggestion that the chemical noise probably originates from the large variety of protonated peptides, protonated fragments of peptides, proteins, and matrix that can be generated in a hot MALDI plume.^{[70],[71],[72],[73]} The chemical noise in MALDI and even in ESI^[74] can be a result of the in-source decay of protonated bio-organic species invariably present in the matrix, pipettes, or sample solutions (e.g., bacterial contamination, dander, or dust). Assuming the peptide-based origin of chemical noise, one can theorize that the overall sensitivity of MALDI method drops as $1/m$. Such a rapid drop in sensitivity with the increase of molecular weight of the peptides/proteins under analysis is known in MALDI. This drop makes it challenging to use MALDI in mass analyses of proteins with $\text{MW} > 20,000$ Da where the limit of detection can be well into a pmole range.^[75] The reason for this drop can be a hydrophobic/hydrophilic mismatch^[76] in matrix-analyte co-crystallization that affects MALDI signals starting at a rather low mass range.^{[77],[78]}

Experiments on pressure reduction

The most intriguing part of these studies was the observation of ion signals after routine acquisition when the UV laser was turned off and the sample was removed from the inlet capillary. First, the sample spot was irradiated for 3–10 seconds to record a routine AP-MALDI spectrum. During the AP-MALDI experiment, the pressure in the high-pressure funnel was kept at 15 Torr and the capillary temperature kept at 165° C. Several seconds after the UV laser was turned off and the target plate was moved into a position containing no sample, the pressure in the ion funnel system was gradually reduced by opening the throttle valve of the fore-vacuum pump evacuating the first funnel. During this time, the

mass spectrometer kept recording spectra (see Fig. 4). At 12.0-Torr pressure (0.15 min reading on the horizontal axis of Fig. 4), an ion signal appeared over low- m/z region, the width of this “active” m/z zone was approximately 200 m/z units (see panel A in Fig. 4). The dominant signal in the spectra was m/z 279 (this peak was also observed in MALDI spectra), which reached its maximum intensity at approximately 11.8 Torr. This signal persisted until the pressure dropped to 11.5 Torr. As the pressure in the funnel system gradually diminished further, ion signals were observed at higher m/z values and over a broader m/z range. At one point, the active zone split in two zones. When the pressure reached 3.5 Torr the active zone reached m/z 2,000 which was the upper limit of the range used in this study (see panel I).

Importantly, the observed “ion chromatogram” (Fig. 4, bottom panel) reflecting the change in spectra comprising appearing and disappearing peaks was dilated or compressed on a time scale dependent whether a slow or fast pace was chosen for the gradual pressure reduction. The pressure change varied from 15 Torr/min to 5 Torr/min. By observing the appearance of dominant m/z peaks at the same pressures but under different scan rates we concluded that it is a certain pressure but not the rate of its reduction that governed the appearance of peaks over specific m/z zones. Moreover, when the pressure inside the funnel was fixed at a value producing a high intensity peak (see, for instance, panel F in Fig. 4.), the ion signal could persist up to 30 seconds. Interestingly, once the pressure-reducing scan had been completed, a gradual pressure increase from 3 to 16 Torr (the reversed scan) did not produce any spectra, not even any chemical noise. The total peak intensities in “pressure reduction scan” spectra slightly diminished when the capillary temperature was increased from 100°C to 165°C. The overall signal was dependent to a certain degree on the number of laser shots made in the MALDI experiment prior to the pressure reduction scan. A several-fold increase was observed when the MALDI sample was irradiated for three seconds compared with 1 second while the difference between 3-s and 10-s irradiation was minor. No signal appeared during the pressure reduction scan after spraying the inlet with a methanol/water solution immediately after the MALDI experiment. In another experiment, the atmospheric side of the capillary was heated with a heat gun for approximately 1 min. After this “heat treatment”, no signal appeared during the pressure reduction scan.

An interesting feature of the pressure scan spectra is the appearance of a high-intensity peak of doubly-protonated pro14-arg at m/z 767.4 (see Fig. 4, panel D). Yet, in the next time interval (see panel E) the height of m/z 767.4 peak rapidly dropped and became much smaller than the height of the adjacent peak with m/z 757.4. Panel G shows significant chemical noise and a lack of high-intensity peaks. The peaks shown in panel G were detected in the spectrum recorded in a routine AP-MALDI experiment; yet, the intensities of those peaks were very low. The bottom panel (it can be conveniently referred to as the total ion current chromatogram, TIC) as well as panels D, E and G demonstrate one more interesting feature of the pressure scan: once the pressure in the funnel dropped below 10 Torr, the ion signals appear in two “active” m/z zones separated by a gap with no signal. This double-hump feature (see bottom panel, time intervals F and H) corresponds to the re-appearance of m/z 1046.5 and m/z 1533.8 signals. This can be explained by the fact the particular m/z region is swiped twice, first by the “high-mass active zone” and then by the “low-mass active zone”. Two “active” zones can be seen in panel D: the low-mass zone

starts at m/z 200 and ends at m/z 550 while the high-mass zone starts at m/z 750 and ends at m/z 850.

Figure 5 shows the mass spectrum and chemical noise (an m/z 820–870 region is shown) obtained by integrating all the mass spectra recorded during the pressure reduction scan. The integrated mass spectrum resembles the one taken in the AP-MALDI experiment. Yet, one can see two prominent differences: the doubly-protonated pro14-arg peak becomes the dominant one, and the low m/z region contains only a few moderate intensity peaks. An interesting observation is that some low m/z peaks in the pressure reduction scan were also detected in AP-MALDI experiments, but those peaks were not dominant in AP-MALDI spectra. The cornerstone of many suggested MALDI mechanisms, the protonated matrix peak at m/z 190, was almost missing in the pressure scan. The most prominent peak in the AP-MALDI spectrum (Fig. 3), corresponding to a protonated matrix dimer (m/z 379), was of moderate intensity in the pressure reduction scan. The signal-to-noise ratios for high intensity peaks (m/z 1046.5 and m/z 1533.8) were found to be 10- to 20-fold higher than the corresponding ratios in the AP-MALDI spectrum. It is worth emphasizing that the AP-MALDI spectrum was recorded at laser energy levels optimized for attaining the highest S/N ratios. It is also important to add that at this time, no optimization of system parameters has been performed during the experiments on the pressure reduction scan. Once the key parameters of this ionization process are determined, it may be optimized to result in better sensitivity or higher throughput.

Figure 6 compares extracted ion chromatograms (EIC) for the m/z 770–772 interval containing chemical noise, the m/z 767–769 interval containing the doubly-charged pro14-arg peak, and the m/z 757–759 interval containing the singly-charged peak of bradykinin fragment 1–7. The first several seconds represent the ion signal in the corresponding m/z intervals recorded during the MALDI experiment, then the gap (laser was turned off and the target was moved away from the capillary inlet, the pressure was kept constant at 15 Torr), and, finally, the pressure reduction scan. By comparing peak areas, one can see that the total number of doubly-charged pro14-arg ions detected during the pressure scan was approximately 20 times higher than the number of the same ions detected in the MALDI experiment. The chemical noise (m/z 770–772 interval was selected as the chemical noise measure) in the vicinity of the doubly-charged peak is significantly reduced compared with the noise detected in the AP-MALDI experiment. Integrating the peak intensities for the m/z 767.4 signal and background noise in the vicinity of the same peak over the corresponding time window (see panel D in Fig. 4), we calculated that the S/N ratio for the doubly-charged pro14-arg peak increased by a factor of 100 compared with that in MALDI spectra.

Either washing the capillary or flash heating the capillary inlet to high temperatures resulted in the disappearance of the pressure-scan signals. So the hypothesis can be made that the origin of the observed signals is related to the condensation of material desorbed from the MALDI plate onto the inner walls of the relatively cold capillary. Another, albeit remote, possibility is that the sample was deposited onto the funnel wall opposite to the inlet capillary. The CHCA matrix melts at 250 °C, much higher than the temperature of the inlet capillary, especially, in its frontal section that stretches 6 mm away from the heating block and experiences air cooling. It is plausible that the observed signal is related to a gradual

detachment of MALDI matrix material from the interior surface of the inlet capillary. This detachment could be caused by the shear mechanical stress produced by the intruding air flow. Matrix detachment similar to that observed in this experiment has been observed in the past.^[79] In our case, the desorbed matrix material probably sticks to the walls near the capillary inlet. Further down the capillary, the gas velocity rapidly increases, providing progressively lower probability for matrix particles to hit the wall and stick there.

The much more challenging question is the source of protonation. The most probable explanation is that the observed signals are due to matrix-assisted hypersonic-velocity impact ionization (MAHVII) that is similar to hyperthermal impact surface ionization. The electron detachment in the latter is substituted with a “milder” process of protonation in the former. Small particles detached from the capillary wall are entrained into the gas flowing through the capillary. In the capillary, the particles are accelerated to a high velocity. When discharged from the capillary into the low-vacuum chamber, the particles cross the funnel and hit the opposite wall. Studies on VKS mentioned in the introduction indicate that submicron particles travel through the Mach disk without noticeable deceleration. In the high-velocity impact with the surface, the micro- or nanoparticles containing analyte and matrix can acquire thermal energy in a time comparable with the laser pulse duration in MALDI: the shock wave whose speed in solid materials exceeds 10^3 m/sec crosses a one-micron particle in a time of one nanosecond. Such an energetic impact would cause total disintegration of the particulate matter into an ensemble of heated clusters. The process that leads to charge separation in the produced clusters could be the above-mentioned fracto-emission.

Perhaps the most intriguing phenomenon was the observation of “active” zones. The funnel wall opposite to the capillary nozzle comprises a row of thin metal strips - the metalized funnel rings with open gaps between them. There is a steep gradient of the RF field near these surfaces. In a hypothetical MAHVII mechanism, the particles hit the metal surface and ions are produced. To be detected, the produced ions should re-enter the internal volume of the funnel. Yet, the ions flying off the funnel rings can be stopped by a pseudopotential RF barrier whose height depends on ion mobility and local gas pressure.^[80] The additional presence of pronounced aerodynamic drag pushing the ions outside the funnel^[81] could impede the motion of ions from the surface of the rings, across the barrier, toward the inner volume of the funnel. Further experiments as well as modeling should elucidate the ion formation mechanism.

A plausible explanation of why ions are trapped for a long time in the high-pressure funnel could be the hypothetical existence of a “stability zone” in some part of the ion funnel. This zone could be created by a dynamic balance of counteracting forces produced by the aerodynamic drag and the electric field. The gas flow pattern in the region surrounding the capillary nozzle and inside the frontal section of the first funnel can be rather complex. The air flow discharged from the nozzle is pumped from the interior of the funnel through the rectangular cut in the funnel’s wall used for orthogonal injection, through the gaps between the electrodes made in the funnel wall opposite to the nozzle, and through two holes made in the metal backplate that covers the axial entry to the first (high-pressure) funnel. It is possible that the air flow could drag the ions (charged clusters) outside the funnel while

electric forces work to keep the ions inside the funnel. Between these counteracting forces, the charged species could be trapped in some sort of eddies or vortices inside the funnel, and their trajectories would be destabilized by changing the background pressure in the system. The observations would require that the vortices could exist only at relatively high pressure and were unstable at low pressure. This could also explain the observation that the ions are detected only when the pressure gradually diminishes.

Conclusion

Studying an AP-MALDI source operating with a triple-stage ion funnel with orthogonal injection has led to the observation of an intriguing phenomenon of delayed ionization. The appearance of MALDI-like ions in the absence of laser irradiation under the condition of gradual pressure reduction in the ion funnel could be explained, in part, by matrix-assisted hypersonic-velocity impact ionization. It is believed that the mechanisms responsible for ion formation in MALDI and MAHVII are very similar. Yet, at first glance, the hypothetical MAHVII seems to be a “softer” ionization technique. This characteristic can be inferred from observation of much stronger signals of the doubly-charged pro14-arg peptide and a more pronounced peak corresponding to the matrix adduct of the same peptide. Understanding the processes taking place during energetic collisions of microparticles generated during the irradiation of MALDI samples, especially charge separation issues, will benefit from molecular dynamics simulations^[82] that consider shattering of heterogeneous clusters and nanoparticles. Further studies on the hypersonic-velocity impact ionization of nano- or submicron particles containing the matrix (proton donor) and analytes with basic groups as well as transmission/trapping of charged species in the ion funnels caused by a combined action of the aerodynamic drag and electric fields would elucidate details of this startling observation of laserless post-ionization.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.

Figure 6.